GENETIC PREDISPOSITION

This invention relates to a method for assessing predisposition to various conditions based upon polymorphisms in a bone sialoprotein gene, a matrix gla protein gene, an osteopontin gene and/or an osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor (OCIF) gene. More specifically, the invention relates to a method of assessing an individual's predisposition to various pathological calcification conditions including osteoporosis and atherosclerosis by screening for these polymorphisms. The method of the present invention is especially useful in determining allelic variations in the human bone sialoprotein gene, the human matrix gla protein gene, the human osteopontin gene and/or the osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor (OCIF) gene thus predicting predisposition to high or low bone mineral density (BMD). The invention also relates to bone sialoprotein (BSP) genes, matrix gla protein (MGP) genes, ostepeopontin (OPN) genes and OPG/OCIF genes containing the polymorphisms and to probes and primers therefor.

Osteoporosis is today one of the most common diseases in individuals over 60 years of age. In America alone it affects an estimated 25 million people with a 5:1 ratio of women to men. This corresponds to approximately 25-30% of people over 60 years of age. In Europe the percentage of people affected by this disease is approximately the same.

At present there is no cure for osteoporosis. However, hormone replacement therapy as well as treatment with bisphosphonates can halt or slow accelerated bone loss. Hence, the sooner such a bone loss can be diagnosed the

better the impact of treatment. It would, accordingly, be of particular advantage to be able to identify individuals predisposed to osteoporosis as early as possible. Since there is a strong genetic component associated with the development of osteoporosis (see below) the identification of genes having an impact on peak bone mass and/or the rate of bone loss would be of enormous help because individuals with genotypes predisposing to osteoporosis could be identified early in their life, leaving plenty of time for preventive measures to be instituted.

From this point of view it is relevant to identify individuals or groups who are predisposed either to have a relatively low peak bone mass or bone mineral density or who are predisposed to a relatively fast rate of loss of bone mass or bone mineral density.

The extent of genetic contribution to bone mineral density and, hence, possibly to osteoporosis became evident more than 20 years ago from twin studies (Smith et al., 1973). Larger BMD and bone width variances were found in dizygotic than in monozygotic twins, indicating a considerable genetic influence on the regulation of bone mass (Smith et al., 1973). Another twin study reported a statistically significant effect of genetic factors on the rate of BMD change (Kelly et al., 1993). Other studies have demonstrated a strong genetic impact on the acquisition of peak bone mass (Gueguen et al., 1995; Lutz & Tesar, 1990). Hence, the genetic component seemingly has an impact on both peak bone mass and the rate of bone loss. Genetic segregation analysis has strongly suggested that bone mass is controlled by several genes, each with modest effects (Gueguen et al., 1995).

Very little is known about the molecular mechanisms and genetics, leading to osteoporosis, even though there has been an intense search for genes influencing bone formation and bone resorption. Within the last five years polymorphisms identified in genes mainly encoding regulatory proteins have been associated with BMD:

1) A vitamin D receptor gene polymorphism identified by Morrison and co-workers (Morrison et al., 1994; WO94/03633) has been subject of numerous publications. Only about half found a significant but weak association and this association may have a stronger link to peak bone mass than rate of bone loss as suggested by Riggs (Riggs, 1997).

2) An estrogen receptor gene polymorphism identified by Kobayashi and co-workers has been associated with low BMD (Kobayashi et al., 1996, US5834200).

3) A polymorphism in the collagen type I α 1 gene identified by Grant and co-workers (Grant et al., 1996; WO9732041) has been associated with rate of bone loss rather than peak bone mass (Uitterlinden et al., 1998).

4) Two polymorphisms in an AT rich minisatellite repeat in the 3′ flank of the interleukin-6 gene - an interleukin which has a stimulatory effect on cells of the osteoclast lineage - have been found to be associated with low BMD (Murray et al., 1997; WO9743446). These polymorphisms seem to influence peak bone mass.

5) Two tandem repeat polymorphisms recently found in the interleukin-1 receptor antagonist gene have been associated with rate of bone loss (Keen et al., 1998; WO9844150). The reason for focusing on this gene was that interleukin-1 is a potent stimulator of osteoclastic bone resorption. Interleukin-1 is stimulated and interleukin-1

receptor antagonist is inhibited in estrogen deficient states, where the associated bone loss can be blocked by treatment with interleukin-1 receptor antagonist, which then seemed to be a good candidate gene for the regulation of postmenopausal bone loss (Keen et al., 1998).

6) A polymorphism in the osteocalcin gene has recently been reported (Dohi et al., 1998). This polymorphism was unable to discriminate BMDs measured in persons with the aberrant allele from BMDs measured in persons with the wild type genotype in a statistically significant manner.

7) Polymorphisms linked to low BMD or osteoporosis have been described in the TGF-ß1 gene, whose protein product is abundant in bone and an important regulator of bone resorption and formation (Langdahl et al., 1997; Yamada et al., 1998; WO97/28280).

8) A polymorphism in the apolipoprotein E gene has been reported to be correlated to low BMD in postmenopausal Japanese women (Shiraki et al., 1997). The reason for focusing on this gene was a report stating that the level of vitamin K which activates osteocalcin through γ-glutamyl carboxylase was related to the apolipoprotein E phenotype (Saupe et al., 1993).

9) WO9705275 discloses use of analysing for an allelic variant in the retinoic acid receptor gene for the prediction of bone density.

The vitamin D receptor gene and the collagen type I 1 gene polymorphisms only seem to be associated with BMD in a fraction of the cohorts examined (the other gene polymorphisms have only been tested on small, national cohorts and most of them are rather peripheral to bone metabolism). Since the genetic influence on the development

of osteoporosis is caused by the inadequate action of multiple genes, this comes as no surprise. Obviously, more genetic polymorphisms with an impact on bone formation/ resorption need to be identified to get a better genetic prediction power valid within a wider geographical area. In this respect, we believe it is important to focus on the promoter regions of genes encoding proteins present in bone for the following reason: Mutations predisposing for osteoporosis must be rather subtle in nature, since they generally resist physical detection for more than 50 years. Mutations affecting the expression of a gene product in stead of its function would be expected to display such subtle effects. Gene expression is controlled by promoters residing upstream of the coding region of a gene. Mutations in these regions could cause an altered gene expression.

The present invention now provides a method of assessing an individual's predisposition to a selected calcification condition status, which method comprises determining the genotype of the promoter of the bone sialoprotein gene, the promoter of the matrix gla protein gene, the promoter of the osteopontin gene, or the promoter of the OPG/OCIF gene or all four or any combination of two or more out of the four promoters.

The calcification condition status for which a predisposition is assessed according to the invention may be having a high or a low peak bone mass (as a future, present or as a past state) or having a high rate or a low rate of bone loss (as a future, present or past state). Thus, the invention may be used to assess a predispostion to osteoporosis.

In a second aspect the present invention provides a method of assessing predisposition of an individual to any

condition associated with allelic variation of a said promoter or any such combination thereof.

The method of the invention typically comprises determining whether an individual is homozygous or heterozygous for a bone sialoprotein promoter (BSP), a matrix gla protein promoter (MGP), an osteopontin promoter (OPN), or an OPG/OCIF promoter or all four promoters or combinations of two or more out of these four promoters and particular polymorphisms thereof. The method is conveniently used to screen for an individual at risk of a condition or disease correlated with aberrant production of bone sialoprotein, matrix gla protein, osteopontin, or OPG/OCIF or all four or two or more out of the four such as osteoporosis or atherosclerosis.

A DNA sequence of the human bone sialoprotein promoter is known and has been published by Kim, R.H. et al. in Matrix Biol. 14: 31-40 (1994). The sequence submitted to GenBank by this group with accession # L24756 is referred to hereafter as the wild type sequence or the published sequence. A DNA sequence of the human matrix gla protein promoter is known and has been published by Cancela, L. et al. in J. Biol. Chem. 265 (25): 15040-15048 (1990). The sequence submitted to GenBank by this group with accession # M55270 is referred to hereafter as the wild type sequence or the published sequence. A DNA sequence of the human osteopontin promoter is known and has been published by Hijiya et al. in Biochem J., 303: 255-262 (1994). The sequence submitted to GenBank by this group with accession # D14813 is referred to hereafter as the wild type sequence or the published sequence. A DNA sequence of the human OPG/OCIF promoter is known and has been published by Morinaga et al., Eur. J. Biochem. 254(3):658-691 (1998).

The sequence submitted to GenBank by this group with accession #AB008821 is referred to hereafter as the wild type sequence or the published sequence. This terminology is not intended to imply that any of these published sequences is more prevalent in the population than variations thereof or that each or any of them is associated with the minimum risk of pathology. The method of the invention includes determining whether the individual being tested has a bone sialoprotein promoter, a matrix gla protein promoter, an osteopontin promoter, or an OPG/OCIF promoter or all four or combinations of two or more out of these four promoters which are identical with the published sequences (or are identical at selected regions of said sequences) or whether that individual has a bone sialoprotein promoter, a matrix gla protein promoter, an osteopontin promoter, or an OPG/OCIF promoter or all four or combinations of two or more out of these four which differ from the published sequences (or which differ at said selected locations), i.e. are polymorphisms of the published sequences, whether homozygous or heterozygous.

The invention includes a method as described above in which one determines the sequence at location 1496 bp of the BSP, in particular whether the sequence at this location is A (published) or G, and/or at the location 1869 bp, and in particular whether the sequence at said location is G (published) or A. The locations identified above are numbered from the start of the published sequence. In an alternative numbering scheme, these locations are -683 bp and -310 bp from the start of the transcribed sequence of the gene. Hereafter, these specific allelic variations are indicated using the terminology BSP-A1496G and BSP-G1869A.

Similarly, the invention includes such a method in which said allelic variation is at MGP location 242 bp (numbered from the start of the published sequence or -3157 bp from the start of the transcribed sequence, and in particular whether the sequence at said location reads C (published) or A, referred to hereafter as MGP-C242A.

Similarly, the invention includes such a method in which said allelic variation is at OPN location 520 bp (numbered from the start of the published sequence or -1748 bp from the start of the transcribed sequence) and in particular whether the sequence at said location reads G (published) or A, referred to hereafter as OPN-G520A.

Similarly, the invention includes such a method in which said allelic variation is at OPN location 1825 bp (numbered from the start of the published sequence or -443 bp from the start of the transcribed sequence) and in particular whether the sequence at said location reads T (published) or C, referred to hereafter as OPN-T1825C.

The invention includes a method as described above in which one determines the sequence at location 163 bp of the OPG/OCIF promoter, in particular whether the sequence at this location is A (published) or G. The location specified above is numbered from the start of the published sequence. In an alternative numbering scheme, this location is -943 bp from the start of the transcribed sequence of the gene. Hereafter, this specific allelic variation is indicated using the terminology OPG-A163G.

We have found that if at least one copy of the BSP gene of the individual has G rather than A at position 1496 or A rather than G at position 1869, this is associated with higher peak bone mass.

Similarly, we have found that if at least one copy of the MGP gene of the individual has A rather than C at position 242, this is associated with a higher rate of loss of bone mass.

We have further found that having at least one copy of A rather than G at OPN position 520 bp is associated with a higher rate of loss of bone.

We have further found that having at least one copy of T rather than C at OPN position 1825 bp is associated with a lower bone mass.

We have found that if at least one copy of the OPG/OCIF gene of an individual has A rather than G at position 163 bp, this is associated with higher bone mass.

The relevant determinations of gene promoter sequences can be carried out by generally known methods, which generally involve amplifying a relevant portion of the DNA of a said gene promoter of said individual. The sequence of said amplified portion may be determined by hybridisation assay or by restriction fragment length analysis.

In particular, amplification may be conducted using a promoter chosen such that if a selected one of the published sequences or the variation of the published sequence is present, amplification will produce a new site at which the amplicon will be cut by a restriction enzyme. A different number of restriction fragments will thus be produced by enzyme treatment of the amplicon. The invention includes an oligonucleotide primer for use in amplification of a relevant portion of a said gene promoter. In particular, the invention includes such a promoter selected so as to produce a different restriction pattern depending on the presence or absence of a selected

variation. Suitable promoters according to the invention are described in the examples hereafter.

The invention includes a method of osteoporosis therapy comprising determining a predisposition as described above, and administering a medicament to the individual to prevent or treat osteoporosis or to delay the onset of osteoporosis if the individual is predisposed to low peak bone mass or to a high rate of loss of bone mass.

Bone sialoprotein is a bone tissue specific 33-34 kDa nascent protein which is extensively modified post-translationally by glycosylation, phosphorylation and sulfation leading to a final MW of 57 kDa (Oldberg et al., 1988; Ecarot-Charrier et al., 1989; Fisher et al., 1990; Zhang et al., 1990). Together with osteopontin, BSP is the most abundant non-collagenous protein in the bone matrix (Nagata et al., 1991). It contains an RGD motif that mediates cell attachment via $\alpha_v\beta_3$ integrin class of cell surface receptor found on osteoclasts (Oldberg et al.1988; Flores et al., 1992; Ross et al., 1993). It also has several segments of poly-glutamic acid that create a potent hydroxyapatite nucleating domain (Hunter & Goldberg, 1993). Temporal studies on the localization of endogenous BSP in rats (Chen et al., 1991; Chen et al., 1992) and in transgenic mice (Chen et al., 1996) have shown, that highest expression of BSP occurs in neonatal bones, with expression decreasing profoundly with subsequent growth and development. BSP has been localized ahead of the mineralization front using immuno-histochemistry techniques, suggesting that it is necessary for the initiation of bone mineralization (Roach, 1994). Accordingly, mRNA has only been detected in differentiated osteoblasts, odontoblasts, and cementoblasts at sites of *de novo*

mineralized tissue formation (Chen et al., 1991; Chen et al., 1992). In transgenic mice, with the transgene being an appr. 2.7 kb promoter region from rat BSP fused to the luciferase gene, the same expression pattern is observed, indicating that the appr. 2.7 kb region of the rat promoter is sufficient to mediate the bone tissue specific transcription (Chen et al., 1996).

Matrix gla protein is a small 79 amino acid residues protein with molecular weight of appr. 14 kDa which contains five γ-carboxyglutamic acid (gla) residues (Price & Williamson, 1985; Loeser & Wallin, 1992). The gla residues are presumably products of a post-translational modification by the vitamin K dependent enzyme γ-carboxylase. MGP strongly binds to hydroxyapatite in a gla dependent fashion (Dowd et al., 1995). High levels of MGP are found in the extracellular matrix of bone, dentin and cartilage (Hale et al., 1988). However, MGP is expressed in many tissues, with the highest levels of mRNA found in lung, heart, kidney and cartilage (Fraser & Price., 1988). A first indication of the function of MGP in bone came from experiments with rats treated with the γ-carboxylase inhibitor warfarin. These animals showed excessive mineralization of the growth plate, indicating that one function of MGP in bone and cartilage could be inhibition of hydroxyapatite formation (Price et al., 1982). Final proof for this function has come from a recent study on MGP knockout mice, which die within 2 months after birth as a result of arterial calcification leading to blood-vessel rupture. MGP deficient mice also display inappropriate calcification of the growth plate cartilage, where calcification has extended into the zone of proliferating chondrocytes rather than being restricted to the lower

hypertrophic zone as observed in normal animals. The abnormal calcification led to a disorganization of chondrocyte columns, eventually resulting in short stature, osteopenia and fractures (Luo et al., 1997). These results strongly suggest that MGP functions to inhibit calcification in soft tissues and restrict mineralization within the growth plate cartilage to the lower hypertrophic zone - the latter possibly by inhibiting calcification in the underlying area of proliferating chondrocytes.

Osteopontin is a phosphorylated and glycosylated protein of 44 kDa (Prince et al., 1987). Together with BSP, OPN is the most abundant non-collagenous protein in the bone matrix (Nagata et al., 1991), but it is, unlike BSP, also expressed in several other tissues (Denhardt & Guo 1993). Osteopontin and BSP are clearly related: 1) they both have an RGD domain that mediates cell attachment via $\alpha_v\beta_3$ integrin class of cell surface receptor (Ross et al., 1993; Wong et al., 1996), and 2) they both have a high content of acidic amino acids (OPN has poly-aspartic acid segments and BSP has poly-glutamic acid segments) and sialic acid (Franzen & Heinegård).

Phosphorylated osteopontin is a potent inhibitor of hydroxyapatite formation while the dephosphorylated form is far less potent (Hunter et al., 1994). In bone osteopontin is found at high concentrations in the lamina limitans that underlies bone lining cells and in reversal (cement) lines found at matrix-matrix interfaces where bone deposition has been preceded by a resorptive event (McKee & Nanci 1996; McKee et al., 1993). These findings together with the hydroxyapatite inhibiting activity of osteopontin suggest that osteopontin may act to seal off growing hydroxyapatite surfaces once active bone formation has ceased, as

speculated by Hunter et al., 1994. Recently, it has been demonstrated that osteopontin knockout mice show normal development and bone structure but osteoclast formation is enhanced *in vitro* (Rittling et al., 1998) and osteoclast numbers are higher in epiphyseal regions in OPN -/- than in wild type mice (Yoshitake et al., 1998). Interestingly, Yoshitake and co-workers also showed that newly formed excessive bone following bone marrow ablation in femur from wild type animals was resorbed after 2 weeks while no bone resorbtion was observed in a similar experiment in OPN -/- mice (Yoshitake et al., 1998). Hence, the increased number of osteoclasts in OPN -/- mice is likely a compensation for the reduced resorbing ability. This substantiates the notion put forth by Hunter and co-workers above, that osteopontin acts to limit growing hydroxyapatite surfaces following bone formation.

Another condition, which has been suggested to be promoted by matrix gla protein and osteopontin is atherosclerosis (Shanahan et al., 1994; Sohma et al., 1994). Employing differential hybridization techniques to screen a cDNA library derived from the aortae of Watanabe heritable hyperlipidemic (WHHL) rabbits, Sohma and co-workers found one clone encoding the matrix gla protein (Sohma et al., 1994). Northern blot analysis of RNA prepared from aortae of WHHL and normal rabbits of various ages indicated that the expression of matrix gla protein mRNA increased in proportion to the progression to atherosclerosis in the WHHL rabbits (Sohma et al., 1994). Additionally, mice lacking matrix gla protein die from aortic calcification (Luo et al., 1997). No atherosclerotic plaques were found in these mice, suggesting that matrix gla protein may instead affect calcification once plaques

are formed. Nevertheless, high levels of matrix gla protein have been located in lipid-rich areas of atherosclerotic plaques (Shanahan et al., 1994). Also, high levels of osteopontin mRNA and protein have been found in necrotic lipid cores and areas of calcification in human atheromatous plaques (Shanahan et al., 1994).

Osteoprotegerin (OPG)/osteolastogenesis inhibitory factor (OCIF) was recently identified independently by two groups as a 380 amino acid residue long glycoprotein with a molecular weight of approximately 55 kD related to the tumor necrosis factor receptor (TNF-R) superfamily (Simonet et al., 1997: Yasuda et al., 1997). Unlike the other TNF-R-like molecules this cytokine receptor lacks a trans-membrane domain (Simonet et al., 1997). Accordingly, OPG/OCIF is a secreted protein appearing as a disulfide linked homodimer with a molecular weight of approximately 110 kD (Simonet et al. 1997).

Initially, mice transgenic for rat OPG/OCIF were found to develop osteopetrosis, which indicated that OPG/OCIF either could function to increase osteoblast-mediated bone formation or to decrease osteoclast-mediated bone resorption (Simonet et al., 1997). In an *in vitro* osteoclast-forming assay recombinant OPG/OCIF was found to be a potent inhibitor of osteoclastogenesis (Simonet et al., 1997). In agreement with this finding OPG/OCIF knockout mice develop osteoporosis, emphasising that OPG/OFIC is an important regulator of postnatal bone mass (Bucay et al., 1998).

During mouse embryogenesis OPG/OCIF is highly expressed in cartilage of developing bones, as well as in several major arteries, the gastrointestinal tract, and skin (Simonet et al., 1997). In the adult animal OPG/OCIF

expression is found in several tissues including heart, brain, lung and liver, which is in contrast to human tissue where OPG/OCIF expression is absent in brain and liver, highly expressed in kidney and detectable in various hematopoietic and immune organs (Simonet et al., 1997). No explanations have been offered on these observations, except that it could be due to true species-specific expression differences (Simonet et al., 1997).

The ligand for OPG/OCIF has also been identified independently by the same two groups, who identified OPG/OCIF. The ligand, called OPG ligand (OPGL) (Lacey et al., 1998) or osteoclast differentiation factor (ODF) (Yasuda et al., 1998), is a TNF-related cytokine, which binds to a hematopoietic progenitor cell committed to the osteoclast lineage and stimulates its differentiation into an osteoclast (Lacey et al., 1998). *In vitro* OPGL/ODF also stimulates mature osteoclasts to resorb bone and recombinant OPGL/ODF injected subcutaneously stimulates bone resorption in mice (Lacey et al., 1998). OPGL/ODF is produced either as a 45 kDa membrane bound protein or as a 31 kDa soluble, secreted C-terminal fragment (Lacey et al., 1998).

OPGL/ODF, which is identical to two previously identified cytokines, TNF-related activation-induced cytokine (TRANCE) (Wong et al., 1997) essential for T-cell activation and receptor activator of NF-kB ligand (RANKL) essential for dendritic cell activation (Anderson et al., 1997), is highly expressed in lymphoid tissues and trabecular bone (Lacey et al., 1998: Yasuda et al., 1998).

Since OPG/OCIF binds to and inhibits the action of OPGL/ODF, these two proteins are seemingly important extra-

cellular regulators of osteoclast development and, thus, eventually bone resorption.

Apart from an osteoporotic phenotype OPG/OCIF knockout mice also displayed a marked calcification of the aorta and renal arteries by 2 months of age (Bucay et al., 1998). Thus, OPG/OCIF inhibits decalcification of the skeleton and at the same time inhibits calcification of certain blood vessels. A similar phenomenon has been observed previously in matrix gla protein (MGP) knockouts (Luo et al., 1997). However, the arterial calcification is more disseminated and pronounced in the MGP knockouts, while the bone loss is more severe in the OPG/OCIF knockouts. Aberrant expression of proteins involved in the prevention of the calcification of blood vessels could, theoretically, be associated with the generation of atherosclerotic plaques. However, no atherosclerotic plaques were found in the OPG/OCIF knockout mice (Bucay et al., 1998), excluding a direct role in the generation of atherosclerosis. Nevertheless, it would not exclude that aberrant expression of OPG/OCIF could be a secondary factor accelerating the formation of an atherosclerotic plaque once the initial lesion has occurred.

The method of assessing an individual, predisposition to osteoporosis or other calcification condition related diseases described above may be combined with measurements of bone mass on a whole body or selected location basis. These include X-ray or ultrasound BMD measurements. The methods described herein may also or instead be combined with measurements of chemical bone resorption markers such as the Crosslaps™ measurement of C-telopeptide fragments of Type 1 collagen or measurements of N-telopeptide fragments of Type 1 collagen in body fluids such as serum or urine.

Each of these types of measurement may be treated as a risk factor to be combined in a weighted manner with the one or more of the others (one of course being a genetic predisposition measurement according to this invention).

In order that the nature of the present invention be more clearly understood, there now follows an example in which reference is made to the Figures shown in the accompanying drawings in which:-

Figure 1 panel A shows the location of the two polymorphisms, called BSP-A1496G and BPS-G1869A, in the bone sialoprotein gene promoter. Panel B shows the location of the polymorphism, called MGP-C242A, in the matrix gla protein gene promoter. Panel C shows the location of the polymorphisms, called OPN-G520A and OPN-T1825C, in the osteopontin gene promoter. The wild type sequences encompassing the four polymorphic sites for all three said promoters are shown with the nucleotide at the polymorphic position in bold and with the substituting nucleotide - also in bold - positioned above the polymorphic site. All nucleotide numbering is relative to base pair number 1, which is the most 5' nucleotide of each of the promoter sequences as published in the GenBank nucleotide database.

Figure 2 shows the time dependence of the mean BMCs measured at the distal arm when grouped into two genotypes for the BSP-A1496G polymorphic site. The BMCs were determined at four time points - 1977, 1979, 1989, and 1995 from each individual out of 133 in the 18 years study. Each point on the curves is the mean BMC for a given year and a given genotype with SEM error bars. The table below the chart lists the actual BMC values determined in 1977, 1979, 1989, and 1995 for the two genotype groups as well as

difference - in percent - between the two genotype groups for each of the four sampling years.

Figure 3 shows the time dependence of the mean BMCs measured at the distal arm when grouped into two genotypes for the BSP-G1869A polymorphic site. The BMCs were determined at four time points - 1977, 1979, 1989, and 1995 from each individual out of 133 in the 18 years study. Each point on the curves is the mean BMC for a given year and a given genotype with SEM error bars. The table below the chart lists the actual BMC values determined in 1977, 1979, 1989, and 1995 for the two genotype groups as well as difference - in percent - between the two genotype groups for each of the four sampling years.

Figure 4 shows time dependence of the mean BMCs measured at the distal arm when grouped into two genotypes for a combination of the BSP-A1496G and BSP-G1869A polymorphic sites. The upper curve represents the combination: BSP-A1496G heterozygous/homozygous polymorphic and BSP-G1869A wild type while the lower curve represents the combination: BSP-A1496G wild type and BSP-G1869A heterozygous/homozygous polymorphic. The BMCs were determined at four time points - 1977, 1979, 1989, and 1995 from each individual out of 133 in the 18 years study. Each point on the curves is the mean BMC for a given year and a given genotype with SEM error bars. The table below the chart lists the actual BMC values determined in 1977, 1979, 1989, and 1995 for the two genotype groups as well as difference - in percent - between the two genotype groups for each of the four sampling years.

Figure 5 shows the time dependence of the mean BMCs measured at the distal arm when grouped into two genotypes for the MGP-C242A polymorphic site. The BMCs were

determined at four time points - 1977, 1979, 1989, and 1995 from each individual out of 133 in the 18 years study. Each point on the curves is the mean BMC for a given year and a given genotype with SEM error bars. The table below the chart lists the actual BMC values determined in 1977, 1979, 1989, and 1995 for the two genotype groups as well as difference - in percent - between the two genotype groups for each of the four sampling years.

Figure 6 shows the time dependence of the mean BMCs measured at the distal arm when grouped into two genotypes for the OPN-G520A polymorphic site. The BMCs were determined at four time points - 1977, 1979, 1989, and 1995 from each individual out of 133 in the 18 years study. Each point on the curves is the mean BMC for a given year and a given genotype with SEM error bars. The table below the chart lists the actual BMC values determined in 1977, 1979, 1989, and 1995 for the two genotype groups as well as difference - in percent - between the two genotype groups for each of the four sampling years.

Figure 7 shows the time dependence of the mean BMCs measured at the distal arm when grouped into two genotypes for a combination of the MGP-C242A and OPN-G520A polymorphic sites. The BMCs were determined at four time points - 1977, 1979, 1989, and 1995 from each individual out of 133 in the 18 years study. Each point on the curves is the mean BMC for a given year and a given genotype with SEM error bars. The table below the chart lists the actual BMC values determined in 1977, 1979, 1989, and 1995 for the two genotype groups as well as difference - in percent - between the two genotype groups for each of the four sampling years.

Figure 8 shows the time dependence of the mean BMCs measured at the distal arm when grouped into two genotypes for the OPN-T1825C polymorphic site. The BMCs were determined at four time points - 1977, 1979, 1989, and 1995 from each individual out of 133 in the 18 years study. Each point on the curves is the mean BMC for a given year and a given genotype with SEM error bars. The table below the chart lists the actual BMC values determined in 1977, 1979, 1989, and 1995 for the two genotype groups as well as difference - in percent - between the two genotype groups for each of the four sampling years.

Figure 9 shows the time dependence of the mean BMCs measured at the distal arm when grouped into two genotypes for a combination of the BSP-G1869A and OPN-T1825C polymorphic sites. The BMCs were determined at four time points - 1977, 1979, 1989, and 1995 from each individual out of 133 in the 18 years study. Each point on the curves is the mean BMC for a given year and a given genotype with SEM error bars. The table below the chart lists the actual BMC values determined in 1977, 1979, 1989, and 1995 for the two genotype groups as well as difference - in percent - between the two genotype groups for each of the four sampling years.

Figure 10 shows the location of the OPG-A163G polymorphism in the OPG/OCIF gene promoter. The wild type sequence encompassing the polymorphic site for said promoter is shown with the nucleotide at the polymorphic position in bold and with the substituting nucleotide - also in bold - positioned above the polymorphic site. All nucleotide numbering is relative to base pair number 1, which is the 5' most nucleotide of the promoter sequence as published in the GenBank nucleotide database.

Figure 11 shows the time dependence of the mean BMCs measured at the distal arm when grouped into two genotypes for the OPG-A163G polymorphic site. The BMCs were determined at four time points - 1977, 1979, 1989 and 1995 from each out of 133 individuals in the 18 years study. Each point on the curves is the mean BMC for a given year and a given genotype with SEM error bars. The table below the chart lists the actual BMC values determined in 1977, 1979, 1989 and 1995 for the two genotype groups as well as difference - in percent - between the two genotype groups for each of the four sampling years.

Figure 12 shows time dependence of the mean BMCs measured at the distal arm when grouped into two genotypes for a combination of the BSP-A1496G and OPG-A163G polymorphic sites. The upper curve represents the combination: BSP-A1496G heterozygous/homozygous polymorphic and OPG-A163G wild type while the lower curve represents the combination: BSP-A1496G wild type and OPG-A163G heterozygous/homozygous polymorphic. The BMCs were determined at four time points - 1977, 1979, 1989 and 1995 from each out of 133 individuals in the 18 year study. Each point on the curves is the mean BMC for a given year and a given genotype with SEM error bars. The table below the chart lists the actual BMC values determined in 1977, 1979, 1989 and 1995 for the two genotype groups as well as difference - in percent - between the two genotype groups for each of the four sampling years.

Figure 13 shows time dependence of the mean BMCs measured at the distal arm when grouped into two genotypes for a combination of the BSP-G1869A and OPG-A163G polymorphic sites. The upper curve represents the combination: BSP-A1496G homozygous polymorphic and OPG-

A163G wild type while the lower curve represents the combination: BSP-G1869A wild type/heterozygous and OPG-A163G heterozygous/homozygous polymorphic. The BMCs were determined at four time points - 1977, 1979, 1989 and 1995 from each out of 133 individuals in the 18 year study. Each point on the curves is the mean BMC for a given year and a given genotype with SEM error bars. The table below the chart lists the actual BMC values determined in 1977, 1979, 1989 and 1995 for the two genotype groups as well as difference - in percent - between the two genotype groups for each of the four sampling years.

Example 1 (18 year study)

Methods

Subjects. One hundred thirty three women followed up for 18 years (1977-1995) with respect to BMD, biochemical markers, height, and weight were used in the study. A detailed description of the cohort has been previously published (Jørgensen et al., 1996).

DNA analyses. Screening for the BSP-A1496G and the BSP-G1869A polymorphisms (basepair numbering according to numbering of BSP promoter sequence submitted to GenBank, accession #L24756), the MGP-C242A polymorphism (basepair numbering according to numbering of MGP promoter sequence submitted to GenBank, accession #M55270), as well as the OPN-G520A and OPN-T1825C polymorphisms (basepair numbering according to numbering of osteopontin promoter sequence submitted to GenBank, accession #D14813) were performed as follows:

The polymerase chain reaction (PCR) was used to amplify approximately 250 bp long DNA fragments of the BSP,

MGP, and OPN promoters encompassing the BSP-A1496G, BSP-G1869A, MGP-C242A, OPN-G520A, and OPN-T1825C polymorphic basepairs. PCR techniques are well known in the art and it would be within the ambit of a person of ordinary skill in this art to identify primers for amplifying a suitable section of the BSP, MGP and OPN genes including the positions 1496bp and 1869bp in the BSP promoter, the position 242bp in the MGP promoter, and the positions 520bp and 1825bp in the osteopontin promoter. PCR techniques are described for example in patents US4683202 or EP0200362B1. Two hundred ng of genomic DNA was added to 25 μl reaction containing 1x Taq polymerase buffer with 1.5 mM $MgCl_2$ (Perkin Elmer), 5 nmol of each dNTP, 20 pmol of forward and reverse primer, and 1.25 units of AmpliTaq Gold (Perkin Elmer). The reaction was heated to 95°C for 9 minutes followed by 35 cycles of 95°C for 30 seconds, 46°C (BSP-A1496G and BSP-G1869A polymorphisms) or 49°C (MGP-C242A polymorphism) or 46°C (OPN-G520A polymorphism) or 48°C (OPN-T1825C) for 30 seconds and 72°C for 30 seconds - the latter incubation with a 5 second time extension per cycle. The reaction was finally incubated 7 minutes at 72°C for completion of the extension reaction. Primer sequences for PCR amplification of DNA fragments encompassing the BSP-A1496G, BSP-G1869A, MGP-C242A, and OPN-G520A and OPN-T1825C polymorphic basepairs were:

BSP-A1496G polymorphism primer set:

Forward primer: 5'- GAA AAG ATA TAT ATA GAA GCC CAA G - 3' (SEQ 1D No. 1)

Reverse primer: 5'- TAA TAT CAT TTG ATG TTT CCT CCT G - 3' (SEQ 1D No. 2)

BSP-G1869A polymorphism primer set:
Forward primer: 5'- TTC TTT CGA CAT AGT GAA AAC ACG T - 3' (SEQ 1D No. 3)
Reverse primer: 5'- CGT GGA TTC TCA CCA GAA AAC - 3' (SEQ 1D No.4)

MGP-C242A polymorphism primer set:
Forward primer: 5'- CAG TGA GAA AGC TCA TCA CTT GGT C - 3' (SEQ 1D No. 5)
Reverse primer: 5'- ATT CTC CCA TCC ATC CAT CCA TGC A - 3' (SEQ 1D No. 6)

OPN-G520A polymorphism primer set:
Forward primer: 5'- CGC TGG AAT TAA GAA AAT TGG TAG A - 3' (SEQ 1D No. 7)
Reverse primer: 5'- GTT GTC AAT TTA GTG GAG GGA GAT C - 3' (SEQ 1D No. 8)

OPN-T1825C polymorphism primer set:
Forward primer: 5'- GAG TAG TAA AGG ACA GAG GCG AGC T - 3' (SEQ 1D No. 9)
Reverse primer: 5' - CTA GCT TTT TCA TTT ACG GGA TGG G - 3' (SEQ 1D No. 10)

To determine the presence or absence of a polymorphic genotype of PCR amplified DNA fragments using the above mentioned PCR primer sets, restriction enzyme analyses were performed as follows:

DNA fragments PCR amplified using the BSP-A1496G polymorphism primer set were restricted with Eco T14 I in a 20 µl reaction containing: 1x buffer H (Amersham Pharmacia), 4 units of Eco T14 I (Amersham Pharmacia) and 5 µl of the cycled PCR reaction. The reaction mixture was incubated at 37°C for 1 hour. Four µl 6xgel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30%

glycerol in water) were added to the 20 µl Eco T14 I digest and loaded on a 2.5% agarose gel. DNA fragments were then resolved by electrophoresis until the bromophenol blue marker had run 2/3 through the gel. If the DNA sample analyzed was homozygous for the wild type BSP sequence one band of 270 bp would be observed. If the DNA sample analyzed was heterozygous two bands of 270 bp and 245 bp would be observed. If the DNA sample was homozygous for the polymorphism one band of 245 bp would be observed.

DNA fragments PCR amplified using the BSP-G1869A polymorphism primer set were restricted with Eco 72 I in a 20 µl reaction containing: 1x Universal buffer (Stratagene), 4 units of Eco 72 I (Stratagene) and 5 µl of the cycled PCR reaction. The reaction mixture was incubated at 37°C for 1 hour. Four µl 6xgel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) were added to the 20 µl Eco 72 I digest and loaded on a 2.5% agarose gel. DNA fragments were then resolved by electrophoresis until the bromophenol blue marker had run 2/3 through the gel. If the DNA sample analyzed was homozygous for the wild type BSP sequence one band of 253 bp would be observed. If the DNA sample analyzed was heterozygous two bands of 253 bp and 230 bp would be observed. If the DNA sample was homozygous for the polymorphism one band of 230 bp would be observed.

DNA fragments PCR amplified using the MGP-C242A polymorphism primer set were restricted with Eco T22 I in a 20 µl reaction containing: 1x buffer H (Amersham Pharmacia), 4 units of Eco T22 I (Amersham Pharmacia) and 5 µl of the cycled PCR reaction. The reaction mixture was incubated at 37°C for 1 hour. Four µl 6xgel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30%

glycerol in water) were added to the 20 µl Eco T22 I digest and loaded on a 2.5% agarose gel. DNA fragments were then resolved by electrophoresis until the bromophenol blue marker had run 2/3 through the gel. If the DNA sample analyzed was homozygous for the wild type MGP sequence one band of 266 bp would be observed. If the DNA sample analyzed was heterozygous two bands of 266 bp and 241 bp would be observed. If the DNA sample was homozygous for the polymorphism one band of 241 bp would be observed.

DNA fragments PCR amplified using the OPN-G520A polymorphism primer set were restricted with Bgl II in a 20 µl reaction containing: 1x buffer H (Amersham Pharmacia), 4 units of Bgl II (Amersham Pharmacia) and 5 µl of the cycled PCR reaction. The reaction mixture was incubated at 37°C for 1 hour. Four µl 6xgel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) were added to the 20 µl Bgl II digest and loaded on a 2.5% agarose gel. DNA fragments were then resolved by electrophoresis until the bromophenol blue marker had run 2/3 through the gel. If the DNA sample analyzed was homozygous for the wild type OPN sequence one band of 278 bp would be observed. If the DNA sample analyzed was heterozygous two bands of 278 bp and 257 bp would be observed. If the DNA sample was homozygous for the polymorphism one band of 257 bp would be observed.

DNA fragments PCR amplified using the OPN-T1825C polymorphism primer set were restricted with Sac I in a 20 µl reaction containing: 1x buffer L (Amersham Pharmacia), 4 units of Sac I (Amersham Pharmacia) and 5 µl of the cycled PCR reaction. The reaction mixture was incubated at 37°C for 1 hour. Four µl 6xgel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) were

added to the 20 μl Sac I digest and loaded on a 2.5% agarose gel. DNA fragments were then resolved by electrophoresis until the bromophenol blue marker had run 2/3 through the gel. If the DNA sample analyzed was homozygous for the wild type OPN sequence one band of 256 bp would be observed. If the DNA sample analyzed was heterozygous two bands of 256 bp and 235 bp would be observed. If the DNA sample was homozygous for the polymorphism one band of 235 bp would be observed.

Statistical Methods. To test whether the difference between mean BMDs of two genotypes was statistically significant two-way, unpaired t-tests were applied.

Results

Five previously unknown polymorphisms were identified by sequencing specific promoter regions from the human BSP gene promoter, the human MGP gene promoter, and the human OPN gene promoter following a PCR amplification of 40 DNA samples from healthy women. The BSP-A1496G, BSP-G1869A, MGP-C242A, OPN-G520A, and OPN-T1825C polymorphisms were coded as Xx, Yy, Zz, Bb, and Ss, respectively, where the uppercase letter signifies presence of the wild type base pair at the given polymorphic position and the lowercase letter signifies presence of the base pair different from the wild type base pair at the given polymorphic position.

The 133 DNA samples from the 18 year study were screened for the presence of either of the 5 polymorphisms.

Table 1 shows the genotype distribution of DNA samples from the 18 years study for all identified polymorphic sites: BSP-A1496G, BSP-G1869A, MGP-C242A, OPN-G520A, and

OPN-T1825C. The left panel shows the actual number of samples categorized into three genotypes for the 3 identified polymorphic sites. The right panel displays the same analysis as the left except that the numbers represent the percent of total samples analyzed for each polymorphic site.

wt = XX, YY, ZZ, BB or SS

hz = Xx, Yy, Zz, Bb or Ss

pm = xx, yy, zz, bb or ss

Table 1

18 Year Study

Genotype distribution

Number of samples

	Wt	hz	pm	total
BSP-A1496G	67	53	7	127
BSP-G1869A	4	40	84	128
MGP-C242A	43	64	24	131
OPN-G520A	13	50	63	126
OPN-T1825C	41	54	24	119

% of total

	wt	hz	pm	Total
BSP-A1496G	52.8	41.7	5.5	100.0
BSP-G1869A	3.1	31.3	65.6	100.0
MGP-C242A	32.8	48.9	18.3	100.0
OPN-G520A	10.3	39.7	50.0	100.0
OPN-T1825C	34.5	45.4	20.2	100.0

The genotype distributions for the 5 polymorphisms are shown in table 1. For the BSP-A1496G polymorphism the homozygous wild type genotype was the most abundant, followed by the heterozygous and homozygous polymorphic genotypes, with the homozygous polymorphic groups being quite small. In the case of the BSP-G1869A polymorphism, the wild type genotype, as defined by the BSP gene promoter sequence from GenBank, was rare, the heterozygous genotype was 10 times more frequent and the homozygous polymorphic genotype was twice as frequent as the heterozygous. For the MGP-C242A polymorphism the heterozygous genotype was the most abundant followed by the homozygous wild type and homozygous polymorphic genotypes. In the case of the OPN-G520A polymorphism, the homozygous polymorphic genotype was the most abundant followed by the heterozygous and the wild type homozygous genotypes. The genotype distribution of the OPN-T1825C polymorphism was, generally, the same as for the MGP-C242A polymorphism.

The impact of the 5 identified polymorphic sites on bone mass as represented by BMC and BMD measurements at the distal arm, as well as percent change of BMC/BMD over time, was analyzed. A compilation of these analyses is shown in table 2.

Table 2 shows a compilation of a statistical analysis of the results obtained from screening of the DNA samples from the 18 years study for the presence of either of the 5 identified polymorphisms. Panel A: The numbers represent the likelihood that the difference in mean BMD between groups of different genotype (homozygous wild type or heterozygous/homozygous polymorphic) are identical. The test includes BMC (bone mineral content) and BMD measured at the distal arm. Numbers in parentheses represent the year of BMC/BMD measurement. Panel B: The numbers represent the difference between the mean BMDs of two genotype groups (heterozygous/homozygous polymorphic group subtracted from the homozygous wild type group) in percent of the highest BMD for a given polymorphic site.

Table 2

A)

t-test, p-values for "wt = hz+pm genotype" hypothesis

	BSP-A1496G	BSP-G1869A	MGP-C242A	OPN-G520A	OPN-T1825C	BSP-A1496G/ BSP-G1869A	MGP-C242A/ OPN-G520A	BSP-G1869A/ OPN-T1825C
BMC, arm (77)	0.057	0.079	0.663	0.931	0.98	0.023	0.867	0.056
BMD, arm (95)	0.008	0.015	0.481	0.104	0.83	0.003	0.128	0.1079

B)

BMC and BMD percent differences between genotype groups.

	BSP-A1496G	BSP-G1869A	MGP-C242A	OPN-G520A	OPN-T1825C	BSP-A1496G/ BSP-G1869A	MGP-C242A/ OPN-G520A	BSP-G1869A/ OPN-T1825C
BMC, arm (77)	5.4	5	1.3	0.2	0.1	8.3	0.7	7.6
BMD, arm (95)	9.7	8.3	2.5	5.5	1.6	13.8	6.6	10.5

From this table it is clear that the BSP-A1496G and BSP-G1869A polymorphic sites, especially when combined, are good sites for predicting whether an individual is genetically predisposed for high or low BMC/BMD. The OPN-T1825C polymorphism only has a marginal influence on BMC/BMD on its own. However, when the OPN-T1825C polymorphism is combined with the BSP-G1869A polymorphism the percent separation of genotypes is better than either polymorphism alone (Table 2). On the other hand, the MGP-C242A and OPN-G520A polymorphisms are, at first glance, not suitable sites for such a prediction. None of the identified polymorphisms appeared to have a statistically significant impact on the change in bone mass over time (data not shown). Age, height and weight of the individuals involved in the 18 years study did not differ significantly between any of the genotype groups (data not shown).

These observations strongly indicated that the BSP polymorphisms influence peak bone mass rather than the rate of bone loss. To substantiate this an analysis of the variation of BMD as measured 4 times on the same individual from 1977 to 1995 for the different genotypes was performed. In 1977 the average age of the individuals included in the 18 years study was 51.1 years, thus ending at 69.1 years in 1995. The expected outcome of a plot of the means of BMD for one of the two BSP polymorphisms as a function of time would be two parallel curves, each representing BMDs measured in individuals with the wild type genotype and BMDs measured in individuals with the polymorphic phenotype. Figures 2 and 3 show that this is, indeed, the case for the BSP-A1496G and BSP-G1869A polymorphic sites. Moreover, the two BSP promoter

polymorphisms act in concert on peak bone mass to augment the mean BMD difference between genotypes even more than the isolated contribution of each polymorphism (Figure 4).

The role - if any - of the MGP-C242A and OPN-G520A polymorphisms in the MGP and OPN promoters on bone turnover was less clear from the first analyses compiled in table 2. However, when BMC values grouped according to genotype were plotted as a function of time a set of curves appeared suggesting that both the MGP-C242A (Figure 5) and OPN-G520A (Figure 6) polymorphic sites are determinants of rate of bone loss. It is especially noteworthy that the ZZ and Zz+zz curves as well as the BB+Bb and bb curves separate between 1979 and 1989, corresponding to an average age of 53.1 years and 63.1 years, indicative of a genetic phenomenon associated with the menopause. Like the BSP polymorphisms, the combined action of the MGP-C242A and OPN-G520A polymorphisms also leads to a bigger difference between genotypes than either would create alone (Figure 7).

According to the results compiled in table 2, the impact of the OPN-T1825C polymorphism on BMC/BMD was only visible after it was combined with the BSP-G1869A polymorphism. From a graph of BMC values grouped according to genotype and plotted as a function of time it is difficult to tell whether this polymorphism has an impact on rate of bone loss or peak bone mass, due to the proximity of the curves (Figure 8). However, combining this polymorphism with the BSP-G1869A polymorphism gave rise to a set of time course curves clearly showing that these two polymorphisms cooperate in an additive fashion, and, hence, that the OPN-T1825C polymorphism may influence peak bone

mass, as the BSP-G1869A polymorphism, rather than the rate of bone loss (Figure 9).

Finally, the association between genotype and urinary osteocalcin (N-MID®, Osteometer Biotech A/S) as well as urinary collagen type 1 C-terminal crosslinks (CrossLaps®, Osteometer Biotech A/S) were examined. As expected there was no significant difference between the mean value of either of the biochemical bone turnover markers for the BSP-A1496G and BSP-G1869A polymorphic sites (data not shown). Also, no significant difference was observed between the mean value of the biochemical bone turnover markers for the MGP-C242A and OPN-G520A polymorphic sites. This is likely due to the equally paced bone loss of the ZZ and Zz+zz genotype groups as well as the BB+Bb and bb genotypes groups at the time of N-MID® and CrossLaps® measurement (1995) according to Figures 5 and 6.

Example 2 (18 year study)

Methods

Subjects. One hundred thirty three women followed up for 18 years (1977-1995) with respect to bone mineral content (BCM) or bone mineral density (BMD), biochemical markers, height, and weight were used in the study. A detailed description of the cohort has been previously published (Jørgensen et al., 1996).

DNA analyses. Screening for the OPG-A163G polymorphism (basepair numbering according to numbering of OPG/OCIF promoter sequence submitted to GenBank, accession #AB008821), was performed as follows:

The polymerase chain reaction (PCR) was used to amplify a 253 bp long DNA fragment of the OPG/OCIF promoter encompassing the OPG-A163G polymorphic basepair. Two hundred ng of genomic DNA was added to 25 µl reaction containing 1xPCR Gold buffer (Perkin Elmer), 1.5 mM $MgCl_2$,5 nmol of each dNTP, 20 pmol of forward and reverse primer, and 1.25 units of AmpliTaq Gold (Perkin Elmer). The reaction was heated to 95°C for 9 minutes followed by 35 cycles of 95°C for 30 seconds, 46°C for 30 seconds and 72°C for 30 seconds - the latter incubation with a 5 second time extension per cycle. The reaction was finally incubated 7 minutes at 72°C for completion of the extension reaction. The primer sequences for this PCR amplification were:

OPG-A163G polymorphism primer set:

Forward primer: 5'-AGT CTA ACT TCT AGA CCA GGC AAT T-3' (SEQ 1D No. 11)

Reverse primer: 3'-AGT TAG AGC CAG AGA GAA TCT G-3' (SEQ 1D No. 12)

To determine the presence or absence of a polymorphic genotype of PCR amplified DNA fragments using the PCR primer sets above, restriction enzyme analyses were performed as follows:

DNA fragments PCR amplified using the OPG-C163A polymorphism primer set were restricted with Mfe I in a 20 µl reaction containing: 1 x NEBuffer 4 (New England Biolabs), 4 units of MfeI (New England Biolabs) and 5 µl of the cycled PCR reaction. The reaction mixture was incubated at 37°C for 1 hour. Four µl 6xgel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) were added to the 20 µl Mfe I digest and

loaded on a 2.5% agarose gel. DNA fragments were then resolved by electrophoresis until the bromophenol blue marker had run 2/3 through the gel. If the DNA sample analysed was homozygous for the wild type OPG/OCIF sequence one band of 253 bp would be observed. If the DNA sample analysed was heterozygous two bands of 253 bp and 232 bp would be observed. If the DNA sample was homozygous for the polymorphism one band of 232 bp would be observed.

Statistical Methods. To test whether the difference between mean BMDs of two genotypes was statistically significant two-way, unpaired t-tests were applied.

Results

A previously unknown polymorphism, OPG-A163G, was identified by sequencing the promoter region from the human OPG/OCIF gene promoter, following a PCR amplification of 40 DNA samples from healthy women. The OPG-A163G polymorphism was coded as Mm, where the uppercase letter signifies presence of the wild type base pair at the given polymorphic position and the lowercase letter signifies presence of the base pair different from the wild type base pair at the given polymorphic position. The position of the polymorphism is depicted in Figure 10.

The 133 DNA samples from the 18 year study were screened for the presence of the OPG-A163G polymorphism. The genotype distribution of DNA samples from the 18 year study was as follows: Homozygous wild type (MM) = 71.3% (n=92), heterozygous (Mm) = 25.6% (n = 33), and homozygous polymorphic (mm) = 3.1% (n = 4). The genotype could not be determined in 4 out of the 133 DNA samples.

The impact of the identified polymorphic site on bone mass as represented by bone mineral content (BMC) and bone mineral density (BMD) measurements at the distal arm in 1977 and 1995, respectively, was analysed (Table 3). The percent difference between the genotype groups did not change significantly from 1977 to 1995, which implied that the OPG-A163G polymorphism exerted an influence on peak bone mass. The two polymorphisms, called BSP-A1496G and BSP-G1869A, described above also have an impact on peak bone mass. Hence, it was of interest to examine whether any co-operation between the OPG polymorphism and either of the BSP polymorphisms existed. The combinations OPG-A163G/BSP-A1496G and OPG-A163G/BSP-G1869A showed that those polymor-phisms certainly act in a co-operative fashion, in that the t-test p-values for the null-hypothesis (i.e. no difference between the genotype groups) dropped to statistically significant values and the percent difference in mean BMC/BMD values for two genotype groups increased (Table 3).

Table 3

A)

t-test, p-values for the "wild type genotype group = heterozygous + homozygous genotype polymorphic groups" hypothesis.

	OPG-A163G	OPG-A163G/ BSP-A1496G	OPG-A163G/ BSP-G1869A
BMC, arm (77)	0.022	0.003	0.056
BMD, arm (95)	0.281	0.018	0.030

B)

BMC and BMD percent differences between genotype groups.

	OPG-A163G	OPG-A163G/ BSP-A1496G	OPG-A163G/ BSP-G1869A
BMC, arm (77)	6.7	12.3	8.7
BMD, arm (95)	4.2	13.3	12.3

Thus, it is clear that the OPG-A163G polymorphism, especially in combination with the BSP-A1496G and BSP-G1869A polymorphisms, is a good site for predicting whether an individual is genetically predisposed for high or low BMC/BMD. Age, height and weight of the individuals involved in the 18 year study did not differ significantly between any of the genotype groups (data not shown).

To substantiate the initial indication, that the OPG/OCIF polymorphism influences peak bone mass, an analysis of the variation of BMC as measured 4 times on the same individual from 1977 to 1995 for the different genotypes was performed. In 1977 the average age of the individuals included in the 18 year study was 51.1 years,

thus ending at 69.1 years in 1995. The expected outcome of a plot of the BMC means for the OPG-A163G polymorphism as a function of time would be two parallel curves, each representing BMCs measured in individuals with the wild type genotype and BMCs measured in individuals with the heterozygous or polymorphic homozygous phenotypes. Figure 11 shows that this is certainly the case. Moreover, the combination of the OPG-A163G and the BSP-A1496G polymorphisms show that they act in concert on peak bone mass to augment the mean BMD difference between genotypes even more than the isolated contribution of each polymorphism (Figure 12). In fact, this co-operation is completely additive (Table 4), indicating that the two polymorphisms act on bone mass independently of one another. The numbers for the BSP-A1496G and BSP-G1869A polymorphisms in Table 4 are from the results presented in Figures 2 and 3. Also, the combination of the OPG-A163G and BSP-G1869A polymorphisms indicates a positive co-operation (Figure 13), which is almost additive (Table 4).

Table 4

% difference between the "high BMD" and "low BMD" genotype groups.

	1977	1979	1989	1995
BSP-A1496G	5.1	8.3	7.0	8.5
BSP-G1869A	5.0	6.2	8.8	8.3
OPG-A163G	6.7	9.2	4.6	4.9
OPG-A163G/BSP-A1496G	12.3	17.8	12.7	13.5
OPG-A163G/BSP-G1869G	8.7	12.8	13.0	12.7

Finally, the association between genotype and urinary osteocalcin (N-MID®, Ostometer Biotech A/S) as well as urinary collagen type 1 C-terminal crosslinks (CrossLaps®, Osteometer Biotech A/S) were examined. The urine samples were all collected post the age of attainment of peak bone mass. No significant difference between the mean value of either of the biochemical bone turnover markers for the OPG-A163G polymorphism was found (data not shown). This result is not surprising for a polymorphism with an effect on peak bone mass, due to the equally paced bone loss of the MM and Mm+mm genotype groups at the time of N-MID® and CrossLaps® measurement (1995).

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